International Journal of New Technology and Research (IJNTR)
ISSN:2454-4116, Volume-3, Issue-11, November 2017 Pages 66-70

In-house Production Method for DNA Ladders to Determine Nucleotide Fragment Sizes up to 1500 Base Pairs

Tengis A[1*], Duuriimaa.O[2*], Badamsuren B[2], Batchimeg N[3], Ulziijargal G[3], Tsendmaa TS[1], Javkhlan B[1], Baigalmaa B[1], Bilegtsaikhan TS[1], Munkhtulga L[1], Nyambayar D[1], Munkhbat B[1] Baatartsogt O[2] and Purevjargal N[1]

***Abstract* — the human genome project was recently completed after running for 15 years and revealed the presence of 30,000 genes in the human genome with a total nucleotide length of 3.2 billion base pairs (bp). Many novel methods and techniques have been developed in the field of molecular biology and molecular genetics as a result of intensive research, where basic analysis is impossible without the use of DNA size markers or DNA ladders. This research aimed to establish an in-house method to produce DNA size markers detecting up to 1500 bp size. DNA size markers are commonly used consumables in molecular biology laboratories. In this study, we report preparation of a DNA size marker consisting of 12 fragments from 100 to 1500 bp. DNA fragments were amplified by PCR and PCR products were then ligated in the cloning vector pDYNE TA V2. Our procedure for DNA size marker production could be simple, time saving, and inexpensive.**

***Index Terms*— DNA ladder, transformation, vector, plasmid DNA, cloning**

I. INTRODUCTION

DNA, RNA, and protein molecular weight markers are widely used during separation of the respective molecules by agarose gel electrophoresis and polyacrylamide gel electrophoresis. DNA size markers are used to identify the size of the clone in base pairs after restriction enzyme digestion, to compare the sizes of inserts in PCR screening, to ascertain the sizes of PCR products and during cloning, knock-out, knock-in, and knock down experiments. Most markers are purchased from biotechnology companies but they are very expensive and actual production of markers needs time.

Previously, DNA size markers prepared from the lambda phage [1-4], bacteriophage, simian virus 40 [5], plasmids [6-8] and *Bacillus anthracis* strain [9] were routinely generated by cleavage of the native DNA with restriction enzymes in the laboratory. Commercially, a broad range of DNA ladders from numerous suppliers are available in the market with relatively high prices. This study aimed to produce a DNA ladder in house at a cheaper price.

Here, we describe a method to produce a 100 bp DNA size marker, which minimizes the experimental disadvantages mentioned above. Based on our protocol, any laboratory can prepare its own 100 bp DNA size marker.

Tengis.Altankhuyg, Master student at Core laboratory, Science Technology Center of Mongolian National University of Medical Sciences, Ulaanbaatar, Mongolia
Duuriimaa Otgonbayr, Master at School of Animal Science and Biotechnology, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia
Badamsuren Batsukh, Ph.D student at School of Animal Science and Biotechnology, Mongolian University of Life Sciences, Ulaanbaatar, Mongolia

II. MATERIALS AND METHODS

The cloning vector pDyne TA V2, Dyne Agarose STAR, Dyne PCR purification kit, Dyne Plasmid mini prep, Exiprep Plasmid SV midi prep, TA cloning kit, Dyne Staining STAR, and DH5α competent cells were purchased from DYNEBIO (Korea). Both LB agar and LB broth were purchased from BD Difco (Korea). The general PCR reaction cocktail (GoTaq master mix) was purchased from Promega (USA). The restriction enzymes used were bought from Takara and Toyobo (Osaka and Tokyo, Japan). Oligonucleotides were synthesized by Bioneer (Korea) and all other reagents used were from MonVetMed (Mongolia) unless otherwise noted.

A.PCR Primer design

Oligonucleotide primers were designed to amplify the human peroxidase (PXDN) gene. Peroxidasin (PXDN) was first identified in *Drosophila*, where its mRNA is expressed in ECM-synthesizing hemocytes in the early phase of embryogenesis [10]. PXDN was proposed to function in ECM consolidation, phagocytosis, and defense. PXDN is distinct from other peroxidases; besides its peroxidase domain, it also carries several ECM motifs including immunoglobulin C-2-type domains (IgC2), leucine-rich repeats (LRR), and a von Willebrand factor type C (vWC) [10, 11]. Human PXDN was originally identified as one of the p53-responsive genes in a human colon cancer-cell line [12] and as one of the melanoma-associated antigens [13]. Later, human PXDN (also named vascular peroxidase 1) was characterized as a heme-containing peroxidase with very low enzymatic activity compared with that of other peroxidases [11, 14]. Primer design and sequences were according to those previously described at http://bioinfo.ut.ee/primer3-0.4.0/primer3/. We designed primers against 12 fragments with precise lengths of 100,

200, 300, 400, 500, 600, 700, 800, 900, 1000, 1200 and 1500 bp (Table 1).

Table 1. Primer sequences for PCR amplification

№	Base Pairs (bp)	Sequence	Template	GC %
1F	100	TCCGAGGGATCCTTACACAG	60.07	55.00
1R		GACCATCAAGCCATGCTGTA	59.68	50.00
2F	200	TCAGGTACTTCGGGTCTCCA	60.65	55.00
2R		GTATGTAAAGCCCGCCAGAA	60.10	55.00
3F	300	AGACGCAGGTCGCTATGAGT	60.04	55.00
3R		CTGAATGAGCTGCAATGTCC	59.4	50.00
4F	400	GATCTCCTGGACGAGAGGTG	59.79	60.00
4R		GACAGCCTGGCATTCGTACT	60.29	55.00
5F	500	CTGGGAGAGTACACGGCTA	60.27	60.00
5R		CAGGTCGATGTTGAGTGTCG	6031	55.00
6F	600	TCCCACCCTACCACGACTAC	59.84	60.00
6R		TGAGATGTTCCCCCTGTCTC	60.05	55.00
7F	700	GCTTGTCAACCCACTGCTTT	60.30	50.00
7R		GTCTACCCTGGGGATCTCGT	60.34	60.00
8F	800	ACAAGATCACCTCCCCCTTC	60.31	55.00
8R		CACTGGGTATTTTCCGTGGT	59.71	50.00
9F	900	GTCGTTATTGAGGGCCAGAC	59.56	55.00
9R		TCCGGTACTTCTGGTGGAAG	60.10	55.00
10F	1000	GGAGATCCAGCACATCACCT	60.08	55.00
10R		GGGTATTTTCCGTGGTCTTG	59.29	50.00
11F	1200	TCCGAGGGATCCTTACACAG	60.07	55.00
11R		GTTGAAGATGCCAGCATTGA	59.81	45.00
12F	1500	CCATCACCTGGAACAAGGAT	59.78	50.00
12R		GGTCGTAGCCGTGGTACTCT	59.24	60.00

B. Polymerase Chain Reaction (PCR)

The reaction mix was prepared with the primers described above and Gotaq polymerase and subjected to PCR. The reaction was subjected to 5 min at 94°C (Initial denaturation), 30 cycles of 30 s at 94°C (denaturation), 30 s at 56–60°C (annealing), and 30 s at 72°C (extension), followed by a final extension for 10 min at 72°C as illustrated in Table II. The PCR products were analyzed by electrophoresis (1% agarose gels) in 40 mM Tris-acetate containing 1 mM EDTA (1x TAE) (Table 2).

Table 2. PCR amplified condition

PCR cycle		Temperature	Time
1 Cycle	Initial denaturation	94°C	5 min
30 Cycle	Denaturation	94°C	30 sec
	Annealing	56°C–60°C	30 sec
	Extension	72°C	30 sec
1 Cycle	Final extension	72°C	10 min

C. PCR product purification

The PCR products were transferred to 1.5 ml microcentrifuge tubes. PCR purification buffer was added and mixed by tapping (PCR purification buffer: PCR Sample = 5:1). The spin column was inserted into the collection tube, and the mixture was applied to the column and centrifuged at 13000 rpm for 1 min at room temperature. The flow-through was discarded and the column re-inserted into the collection tube, followed by addition of 750 µl Wash buffer to the column, and centrifuged for 1 min. The flow-through was again discarded, and the column was centrifuged for 1 min at 13000 rpm to remove the residual Wash buffer completely. The spin column was then placed in a clean 1.5 ml microcentrifuge tube and 50 µl of elution buffer was added to the center of the spin column and centrifuged for DNA elution.

D. Ligation

DNA ligation involves formation of a phosphodiester bond between the 3′ hydroxyl group of one nucleotide and the 5′ phosphate group of another. The PCR fragments were ligated into the pDYNE TA V2 cloning vector. The ligation reaction was incubated at 16°C for 5 min (Table 3).

Table 3. Ligation reaction

Dyne 6x buffer	1 µL
pDyne TA-Blunt V2	1 µL
PCR product	2 µL
Distilled water	2 µL
Room temperature 5 min	

E. Transformation of E. coli cells and screening of positive clones by colony PCR

All cloning procedures were carried out using the *E. coli* strain DH5α. For transformation, we first mixed both the PCR fragments and the linear vector in a 10 µL volume of distilled water. This volume was transferred to a microcentrifuge tube containing 50 µL of thawed competent cells. The mixture was incubated for 30 min on ice and then placed in a water bath at 42°C for 1 min immediately after the heat treatment, cells were placed on ice for 10 min. For outgrowth, 80 µL of SOC medium was added to the *E. coli* cells, which were allowed to recover at 37°C for 1 h. Next, 100 µL of transformed cells were plated on solid LB medium containing ampicillin (100µg/ml) as the selective antibiotic. Cells grew overnight at 37°C and the number of colonies was counted on the next day. To test for positive clones, cellular material from 6–12 randomly chosen colonies was transferred to PCR tubes containing 20 µL of H_2O. The tubes were boiled at 96°C for 10 min to lyse the cells and release the plasmid DNA into the solution. Of this solution, 5 µL was used to amplify the inserts over 30 cycles of PCR. Positive clones were verified by agarose gel electrophoresis. Plasmid DNA was then purified from the selected positive clones.

F. Construction of 100-1500 bp fragments

Construction of the 100–1500 bp plasmid DNA was performed by amplifying fragments from the human PXDN gene and then ligating to the pDYNE TA V2 cloning vector. This cloning vector is 3.8 kb in size and contains a pUC origin and a multi-cloning site (MCS). This vector also contains a kanamycin and ampicillin resistance marker. It was named as the pDYNE TA-100 bp plasmid vector. The other 11 fragments were constructed similarly. In this study, we constructed pDYNE TA-100 bp, pDYNE TA-200 bp, pDYNE TA-300 bp, pDYNE TA-400 bp, pDYNE TA-500 bp, pDYNE TA-600 bp, pDYNE TA-700 bp, pDYNE TA-800 bp, pDYNE TA-900 bp, pD

YNE TA-1000 bp, pDYNE TA-1200 bp, and pDYNE TA-15 00 bp.

G. Plasmid DNA purification

Bacterial genomic material was prepared using the Dyne plasmid kit (Product No: 501507216 Korea) according to the manufacturer's instruction. In brief, 250 µl of cell resuspension buffer was added to 1 ml of bacterial pellets cultured overnight in LB broth in a microcentrifuge tube. Then, 250 µl of cell lysis buffer was added and the tube was inverted 4–6 times. Next, 350 µl of neutralization buffer was added and the mixture was centrifuged at 13000 rpm for 10 minutes. The supernatant was transferred to a spin column and centrifuged again at 13.000 rpm for 1 minute. The flow-through was discarded and the column placed back into the collection tube, followed by addition of 500 µl denaturation buffer, and centrifuged at 13.000 rpm for 1 minute. The next step was DNA washing, and so, 750 µl of washing buffer was added to the column and centrifuged at 13000 rpm for 1 minute. The column was centrifuged again for drying properly. The final step was elution for which 50 µl of elution buffer was added to the center of each column. After adsorption for 1 minute the column was centrifuged at 13000 rpm for 1 minute. The eluted plasmid DNA was stored at -20°C until required.

H. Preparation of DNA size marker

A 5-µL aliquot of each PCR mixture was screened by 2% agarose gel electrophoresis, and the fragment length was estimated by comparing with a DNA marker of known size (GeneDireX), The PCR products from each tube were extracted by phenol/chloroform, precipitated with ethanol, analyzed by UV absorbance at 260 nm, and then mixed thoroughly. A 5-µL aliquot of the prepared marker was electrophoresed, and the band migration pattern or band intensities were determined following ethidium bromide staining. The combined marker was frozen at -20°C.

III. RESULTS AND DISCUSSION

In this study, we produced a 100-bp DNA size marker with 12 fragments ranging from 100 to 1500 bp. Our procedure comprised three steps. In detail, the first step involved PCR primer design (Figure 1A), successful amplification of 100–1500 bp fragments by PCR (Figure 1B), and purification of PCR products using a purification kit (Figure 1C).

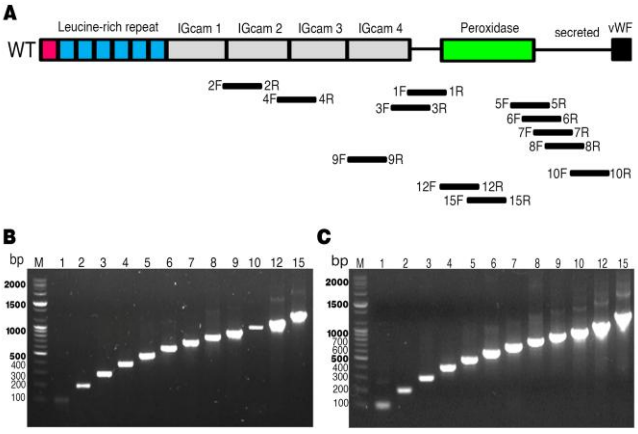

Figure 1. PCR amplification of 100- to 1500-bp DNA fragments. A. Human PXDN gene structure. We designed 12 PCR primers from the IGcam2 domain to the vWF region. 1F: 1R = 100 bp product size marker, 2F: 2R = 200 bp product size marker, 3F: 3R = 300 bp product size marker, 4F: 4R= 400 bp product size marker, 5F: 5R = 500 bp product size marker, 6F: 6R = 600 bp product size marker, 7F: 7R = 700 bp product size marker, 8F: 8R = 800 bp product size marker, 9F: 9R = 900 bp product size marker, 10F: 10R = 1000 bp product size marker, 12F: 12R = 1200 bp product size marker, and 15F: 15R = 1500 bp product size marker. B. Before purification of PCR products. M = size marker; *Lane 1* = 100 bp; *lane 2* = 200 bp; *lane 3* = 300 bp; *lane 4* = 400 bp; *lane 5* = 500 bp; *lane 6* = 600 bp; *lane 7* = 700 bp; *lane 8* = 800 bp; *lane 9* = 900 bp; *lane 10* = 1000 bp; lane 11 = 1200 bp, and lane 15 = 1500 bp products. C. After purification of PCR products. M = size marker; *Lane 1* = 100 bp; *lane 2* = 200 bp; *lane 3* = 300 bp; *lane 4* = 400 bp; *lane 5* = 500 bp; *lane 6* = 600 bp; *lane 7* = 700 bp; *lane 8* = 800 bp; *lane 9* = 900 bp; *lane 10* = 1000 bp; lane 11 = 1200 bp, and lane 15 = 1500 bp products.

The second step was cloning of PCR products which were re-amplified from the self-ligated PCR products. Using the primers designed for the pDYNE TA V2 vector, the largest insert of 1500 bp was quickly selected and recombinant plasmid containing this fragment was named as pDYNE TA-1500 bp (Figue 2A). Using colony PCR, we screened the rate of positive insertion events in 6–12 randomly picked colonies from each of the 12 cloning assays. The results showed that the number of positive clones was proportional to the extent of identity between the insert and the linear plasmid. Colony PCR results showed 90% positive insertion events (Figue 2B).

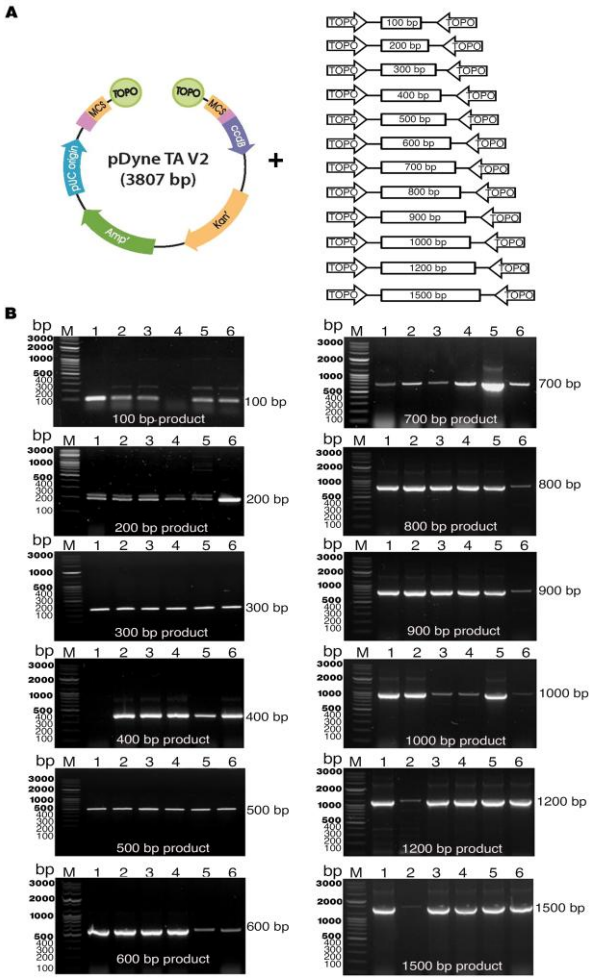

Figure 2. Schematic of pDyne TA -1500 plasmid structure The plasmid contains an insert of 100–1500 bp. A. The 12 PCR products inserted into the pDYNE TA V2 vector can be PCR purified using these 12 different primer pairs. B. Agarose gel electrophoresis of colony PCR products from 12 different amplifications (100–1500 bp), template (human PXDN gene) and annealing temperature range is 56°C–60°C.

In the third step, the purified PCR products were thoroughly mixed, and identified by 1.5% agarose gel electrophoresis. We used different amounts of DNA fragments to prepare the DNA ladder, so that the marker could be easily visible. The prepared DNA marker bands were found to be clear and could be easily observed in a Gel Doc system. We produced three different size markers including a 500 bp ladder (Figue 3A), a 1000 bp ladder (Figure 3B), and a 1500 bp DNA ladder (Figure 3C) in addition to the final DNA size marker (Figure 3D).

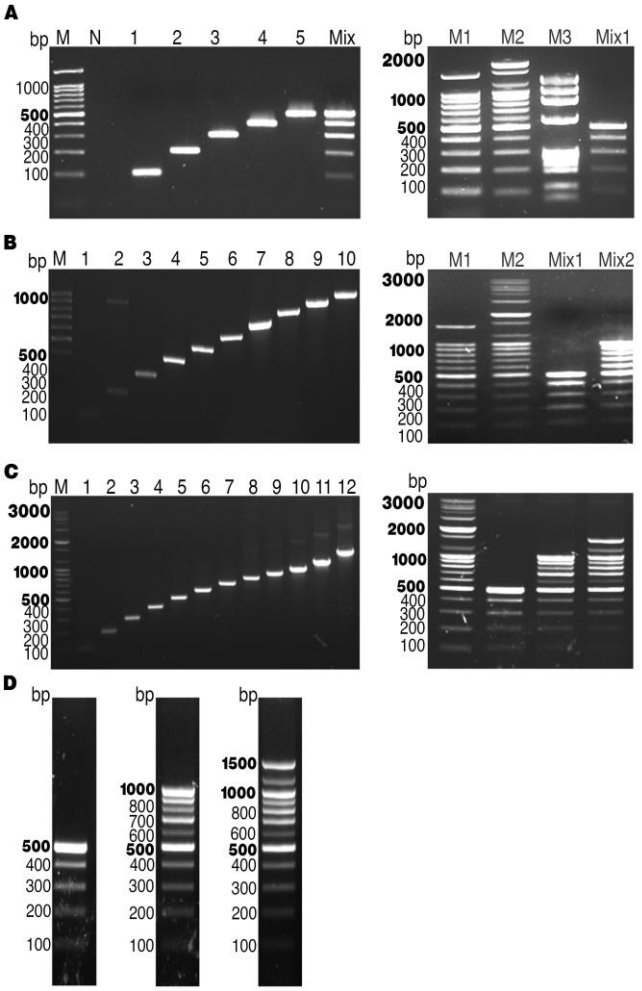

Figure 3. Production of 1500 bp DNA ladder. A. Pattern of 500 bp DNA ladder on 1% agarose gel. The pDyne-500 plasmid was partially designed by producing 5 fragments 100-500 bp in length. M = size marker, N = Negative control, *Lane 1* = 100 bp; *lane 2* = 200 bp; *lane 3* = 300 bp; *lane 4* = 400 bp; and *lane 5* = 500 bp. M1 = DNA size marker (GeneDireX, Cat.No DM001-R500), M2 = DNA size marker (Bioneer Cat No. D-1030), M3 = DNA size marker (Biolabs Cat No: N3026S), and Mix 1= 500 bp DNA ladder (MNUMS). B. Patten of 1000 bp DNA ladder on 1% agarose gel. The pDyne-1000 plasmid was partially designed by producing 10 fragments 100–1000 bp in length. M = size marker, N = Negative control, *Lane 1* = 100 bp; *lane 2* = 200 bp; *lane 3* = 300 bp; *lane 4* = 400 bp; *lane 5* = 500 bp; *lane 6* = 600 bp; *lane 7* = 700 bp; *lane 8* = 800 bp; *lane 9* = 900 bp; and *lane 10* = 1000 bp. M1 = DNA size marker (GeneDireX, Cat.No DM001-R500), M2 = DNA size marker (Bioneer Cat No. D-1030), Mix 1 = 500 bp DNA ladder (MNUMS) and Mix 2 = 1000 bp DNA size marker (MNUMS). C. Pattern of 1500 bp DNA ladder on 1% agarose gel. The pDyne-1500 plasmid was partially designed by producing 12 fragments 100–1500 bp in length. M = size marker, N = Negative control, *Lane 1* = 100 bp; *lane 2* = 200 bp; *lane 3* = 300 bp; *lane 4* = 400 bp; *lane 5* = 500 bp; *lane 6* = 600 bp; *lane 7* = 700 bp; *lane 8* = 800 bp; *lane 9* = 900 bp; *lane 10* = 1000 bp; lane 11 = 1200 bp; and lane 15 = 1500 bp products. M2 = DNA size marker (Bioneer Cat No. D-1030), Mix 1 = 500 bp DNA ladder (MNUMS), Mix 2 = 1000 bp DNA size marker (MNUMS) and Mix 3 = 1500 bp DNA size marker (MNUMS). All the PCR products were combined,

concentrated, and subjected to electrophoresis. Each band contains about 10 ng of DNA, except the 500 bp band, which contained 20 ng. D. The final DNA size marker of up to 1500 bp.

Contribution of authors

M.S performed the laboratory work and prepared the first draft of this paper. D.O and T.A participated in designing the primers and developing the manuscript. B.N, N.N, B.T, and B.B conceived the study and developed the paper. All authors have read this paper and declared no conflict of interest among them.

IV. CONCLUSION

All 12 DNA fragments were easily amplified by the PCR method using *E. coli*, and the DNA size marker could be prepared by mixing the purified PCR products thoroughly. We demonstrated that the in-house prepared DNA size marker was of good quality and could be used as a standard in molecular studies.

Clearly, this strategy was quite straightforward, time saving, and especially inexpensive. For instance, the price of the 100 bp DNA ladder for 75 runs is listed 157 USD (D3687-1VL-Sigma Aldrich), 140 USD (Promega G2101), and 74.9 USD (Bioneer D-1030) on available web sites. We estimated that our protocol requires only 40 USD for materials to produce a 100 bp DNA ladder for 50 runs.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENTS

We thank professor Young Ae Joe Cancer Research Institute, The Catholic University of Korea, Seoul, Republic of Korea for providing human PXDN plasmid DNA and helpful comments. We also thank Dr. Nyambayar N., REKIN Institute, Japan, for helpful comments on the manuscript. This work was supported by the Science Technology Promotion Fund of Mongolian grants funded by the MNUMS (STC: 2016/04).

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